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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

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Additional inventors are being named on the 0 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

ENDOPLASMIC RETICULUM (ER) STRESS

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Respectfully submitted,

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under

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TITLE: ENDOPLASMIC RETICULUM (ER) STRESS

APPLICANT: FUMIHIKO URANO

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ENDOPLASMIC RETICULUM (ER) STRESS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made at least in part with government support under grant no. R01 DK067493-01 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND

Proteins are required for the body to function properly, as they form the basic building blocks of cells, tissue and organ structures. Protein function typically requires the assumption of proper three-dimensional protein structure, which is determined by the amino acid sequence and a process known as protein folding. Sometimes, protein folding goes awry, and misfolded proteins accumulate in cells, causing or contributing to diseases associated with protein misfolding, including amyloidoses (such as immunoglobulin light chain amyloidosis and Alzheimer's disease), Huntington's disease, Parkinson's disease, adult-onset diabetes mellitus, cirrhosis, emphysema, and the prion encephalopathies, alpha-1-antitrypsin deficiency, haemolytic anemia, familial hypercholesterolaemia, amyotrophic lateral sclerosis (ALS), and cystic fibrosis, as well as numerous others. Conformational diseases can be inherited, usually as dominant traits, or can be induced, as in the case of prions.

Proteins destined for secretion such as insulin and alpha1-antitrypsin are translocated into the endoplasmic reticulum (ER) co-translationally; once there, they undergo highly ordered protein folding and post-translational protein processing. However, in some instances, the sensitive folding environment in the ER can be perturbed by pathophysiological processes such as viral infections, environmental toxins, and mutant protein expression, as well as natural processes such as the large biosynthetic load placed on the ER. When the demand that the load of client proteins makes on the ER exceeds the actual folding capacity of the ER to meet that demand, a condition termed "ER stress" results.

Alpha1-antitrypsin (alpha1-AT) deficiency is an exemplary model of a conformational disease. Alpha1-AT is an abundant serum glycoprotein, secreted by the liver, which normally binds to and inactivates elastase, a protease that degrades elastin and collagen. Elastin and collagen maintain the structure of alveoli, air sacs in the lungs. In alpha1-antitrypsin patients, the deficiency leads to uncontrolled destruction of air sacs in the lungs. This condition is called

emphysema and causes a decrease in respiratory function. Alpha1-AT-deficiency mutations interfere with the folding of alpha1-AT, preventing its secretion from the hepatocyte endoplasmic reticulum (ER). Alpha1-AT deficiency is also the leading cause of inherited liver disease in children, caused by the hepatotoxicity of misfolded alpha1-AT molecules that accumulate in the ER lumen.

Cells respond to the accumulation of misfolded proteins in the ER in several ways, including the "ER overload response" and the "unfolded protein response." The "ER overload response" induces the nuclear transcription factor NF-κB, a mediator of the immune response. In patients with cystic fibrosis, expression of mutant CFTR induces NF-κB expression. NF-kappaB upregulates expression of the inflammatory cytokine IL8. Levels of IL-8 are increased in lungs from patients of cystic fibrosis, and NF-κB was found to be constitutively active in mice in which the wild-type CFTR gene had been replaced with the F508 mutant, supporting the theory that ER stress contributes to the chronic inflammation that often contributes to the high morbidity in cystic fibrosis.

The "unfolded protein response" (UPR), triggered by the presence of misfolded protein in the ER, leads to the activation of the kinase Inositol Requiring 1 (IRE1), inducing transcription of genes encoding chaperones and many components of the secretory pathway, and suppresses protein synthesis through a parallel process. In particular, the presence of unfolded proteins in the ER causes dimerization and trans-autophosphorylation of IRE1 that leads to IRE1 activation. Activated IRE1 splices X-Box Binding Protein-1 (XBP-1) mRNA, leading to synthesis of the active form of transcription factor XBP-1 and upregulation of UPR genes (Nature, 2002 Jan 3; 415(6867):92-6; erratum in: Nature 2002 Nov 14; 420(6912):202). In contrast, prolonged ER stress activates the cell death pathway through IRE1.

SUMMARY

The present invention provides novel methods and reagents for quantifying levels of ER stress. In particular, the methods feature Inositol Requiring 1 (IRE1) and XBP-1 as specific markers for ER stress level. As a marker, XBP-1 offers an advantage because ER stress levels can be quantified by monitoring the splicing of XBP-1 mRNA. Exemplary methods are based on PCR. For these methods, only a small tissue sample or a small number of cells are required.

Thus, in one aspect, the invention provides methods of quantifying endoplasmic reticulum stress (ER stress). The methods include detecting an IRE1 activation level in a cell or biological sample, wherein the IRE1 activation level correlates with ER stress, and quantifying the IRE1 activation level, such that ER stress is quantified. In some embodiments, the IRE1 activation level is determined by detecting an XBP-1 splicing level, e.g., by amplifying a XBP-1 mRNA region which includes a splice site, or portion thereof, e.g., to create a DNA complementary to the region of the XBP-1 mRNA, e.g., a double-stranded cDNA PCR product; detecting the size of the amplified mRNA (e.g., the cDNA), wherein the size is indicative of spliced or unspliced mRNA.

In other embodiments, the amplified mRNA is subjected to restriction enzyme digestion, e.g., Pst I digestion, to facilitate detection of spliced or unspliced mRNA. In some embodiments, the ER stress level is quantified in a cell, e.g., a mammalian cell, e.g., a human cell, e.g., a pancreatic beta cell. In some embodiments, the ER stress level is quantified in a cell extract, e.g., an extract from a cell as described herein.

In a second aspect, the invention provides methods of diagnosing an ER stress disease in a subject, e.g., by quantifying the level of ER stress in a cell or biological sample isolated from the subject according to one of the methods described herein; an increased level of ER stress is indicative of the ER stress disease.

In a third aspect, the invention provides methods of monitoring the progression of an ER stress disease, e.g., diabetes, in a subject. The methods include quantifying the level of ER stress in a cell or biological sample isolated from the subject at sequential time points according to one of the methods described herein, wherein a change in the level of ER stress indicates the progress of the ER stress disease.

In a fourth aspect, the invention includes methods for evaluating the effect of a test compound on ER stress. The methods include providing a providing an ER stress model system (e.g., a system comprising a cell expressing IRE1 and XBP-1, e.g., a cultured cell or animal, e.g., a cell or animal model of an ER stress disease.); optionally, increasing levels of ER stress in the system (e.g., in the cells or at least some of the cells of an animal); contacting the system with a test compound; and evaluating the levels of XBP-1 splicing in the system in the presence and absence of the test compound, wherein an increase in XBP-1 splicing indicates an increase in ER stress, and a decrease in XBP-1 splicing indicates a decrease in ER stress.

In some embodiments, the system is an animal model of an ER stress disease, e.g., an animal model of diabetes (e.g., type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Cystic Fibrosis, familial hypercholesterolaemia and alpha1 antitrypsin deficiency, or cells derived therefrom. Typically an ER stress disease can be induced in an otherwise healthy animal or cells by administering a compound known to cause ER dysfunction, e.g., by administering a sublethal dose of thapsigargin, tunicamycin (e.g., 0.25 – 1 mg/kg tunicamycin), or a proteosome inhibitor, e.g., lactacystin.

In some embodiments, the methods include further selecting those test compounds that substantially reduce ER stress (e.g., as measured by XBP-1 splicing levels) as candidate therapeutic compounds for further evaluation.

In a fifth aspect, the invention includes a kit for quantifying ER stress. The kit can include primers for amplifying a region of XBP-1 mRNA which includes a splice site, or portion thereof, and instructions for use. In some embodiments, the kit also includes a suitable control.

The invention also includes an ER stress signaling pathway assay that includes determining the level of ER stress according to one of the methods described herein.

The term "RNA" or "RNA molecule" or "ribonucleic acid molecule" refers to a polymer of ribonucleotides. The term "DNA" or "DNA molecule" or "deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (*e.g.*, by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA can also be chemically synthesized. DNA and RNA can be single-stranded (*i.e.*, ssRNA and ssDNA, respectively) or multi-stranded (*e.g.*, double stranded, *i.e.*, dsRNA and dsDNA, respectively). "snRNA" or "small nuclear RNA" is single-stranded RNA precursor of mRNA. "mRNA" or "messenger RNA" is single-stranded RNA that specifies the amino acid sequence of one or more polypeptide chains. This information is translated during protein synthesis when ribosomes bind to the mRNA. The term "cDNA" or "complementary DNA" refers to a DNA molecule that has a sequence that is complementary to an mRNA or portion thereof, and can include single or double-stranded molecules, but is typically double-stranded.

The term "endoplasmic reticulum stress" ("ER stress") refers to an imbalance between the demand that the load of client proteins makes on the ER and the actual folding capacity of the

ER to meet that demand. A response that counteracts ER stress has been termed "unfolded protein response" ("UPR").

The term "ER stress disorder" refers to a disease or disorder (*e.g.*, a human disease or disorder) caused by or contributed to by increased ER stress levels. Exemplary ER stress disorders include diabetes (*e.g.*, type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Cystic Fibrosis, familial hypercholesterolaemia and alpha1 antitrypsin deficiency.

The term "protein conformational disease" ("PCD") refers to a disease or disorder (*e.g.*, a human disease or disorder) associated with protein misfolding (*e.g.*, caused by or contributed to by protein misfolding). Exemplary protein conformational diseases include, but are not limited to, those diseases listed in Table 1. Other diseases include some rare forms of juvenile diabetes, such as Wolcott-Rallison syndrome and Wolfram syndrome; inflammatory Bowel disease; and cancers originated from secretory cells (e.g., breast cancer and prostate cancer).

Table 1: Exemplary Protein Conformational Diseases

Disease	Protein involved
Alzheimer's disease	amyloid-β
immunoglobulin light chain amyloidosis	immunoglobulin light chain
Parkinson's disease	alpha-synuclein
diabetes mellitus type 2	amylin
amyotrophic lateral sclerosis (ALS)	Superoxide dismutase (SOD)
haemodialysis-related amyloidosis	L2-microglobulin
reactive amyloidosis	amyloid-A
cystic fibrosis	cystic fibrosis transmembrane regulator (CFTR)
sickle cell anemia	hemoglobin
Huntington's disease	huntingtin
Kreutzfeldt-Jakob disease and related disorders (prion encephalopathies)	PrP
familial hypercholesterolaemia	low density lipoprotein (LDL) receptor
Alpha1-antitrypsin deficiency, cirrhosis, emphysema	Alpha1-antitrypsin (alpha1-AT)
systemic and cerebral hereditary amyloidoses	(ten other proteins)

Various methodologies of the instant invention include steps that involve comparing a value, level, feature, characteristic, property, etc. to a "suitable control," referred to interchangeably herein as an "appropriate control." A "suitable control" or "appropriate control" is any control or standard known to one of ordinary skill in the art that is useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing a methodology of the

invention described herein. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a cell or organism, *e.g.*, a control or normal cell or organism, exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a schematic diagram of the unspliced and spliced mouse XBP-1 mRNAs. The coding regions are boxed, the bZip domain is shaded gray and the 26-base pair nucleotide region processed by IRE1 is colored black. The active form of XBP-1 mRNA (cDNA) loses 26 base pairs by IRE1 processing. Spliced form of XBP-1 mRNA encodes larger and active form of XBP-1 protein. The inactive form of XBP-1 cDNA is smaller than the DNA fragment of active form of XBP-1.

FIG. 1B is a reproduction of a gel stained with ethidium bromide (EtBr) showing the results of RT-PCR analysis done with a primer set encompassing the splice junction of XBP-1 mRNA; PCR products were resolved on 2.5 % agarose gel to separate spliced (active form) and unspliced XBP-1 mRNAs. Wild-type or IRE1 mutant mouse embryonic fibroblast cells were untreated or treated with Tunicamycin (Tm) or Thapsigargin (Tg). Total RNA was prepared at the indicated times. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

FIG. 2A is a schematic diagram of the unspliced and spliced murine XBP-1 mRNAs. The coding regions are boxed, the bZip domain is shaded grey and the 26-base pair nucleotide region processed by IRE1 is colored black. The active form of XBP-1 mRNA (cDNA) loses its Pst I site by IRE1 processing. Spliced form of XBP-1 mRNA encodes larger and active form of XBP-1 protein. The inactive form of XBP-1 cDNA digested by Pst I shows two DNA fragments that are smaller than the DNA fragment of active form of XBP-1 digested by Pst I.

FIG. 2B is a reproduction of a gel stained with ethidium bromide (EtBr) showing Pst I digested XBP-1 cDNA from wild-type or IRE1 mutant cells that were untreated or treated with

Tunicamycin (TM) or Thapsigargin (Tg). Total RNA was prepared at the indicated times. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

FIG. 3 is a reproduction of a gel stained with ethidium bromide (EtBr) showing Pst I digested XBP-1 cDNA from mouse islet cells that were untreated (Control) or treated with 1 mM of dithiothreitol (DTT) for 4 hours. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

FIG. 4 is a reproduction of a gel stained with ethidium bromide (EtBr) showing XBP-1 splicing in MIN-6 cells expressing insulin-2 gene with Akita mutation. Pst I digested XBP-1 cDNA from MIN6 cells untransfected (Control), transfected with wild-type Insulin 2 expression vector (Ins2 WT) or with insulin-2 containing Akita mutation expression vector (Ins2 Akita).

FIG 5 is text illustrating the mRNA (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences for the spliced form of XBP-1. The underlined regions of the sequence correspond to (or are reverse complements of) primers (SEQ ID NOs:8 and 9) for amplifying a region of the human XBP-1 mRNA that includes a splice junction. The splice junction is between nucleotides 506 and 507. The bold, underlined regions of the amino acid sequence is the sequence of the C-terminal portion of the protein encoded by the spliced form (SEQ ID NO: 6) that differs from that encoded by the unspliced form, which is bold and underlined in Figure 6.

FIG 6 is text illustrating the mRNA and amino acid sequences for the unspliced form of XBP-1. The underlined regions of the sequence correspond to (or are reverse complements of) primers (SEQ ID NOs:8 and 9) for amplifying a region of the human XBP-1 mRNA that includes a splice junction. The boxed region of the nucleotide sequence is the sequence spliced out by IRE1 (SEQ ID NO: 5). The splice junction is between nucleotides 506 and 507 in Figure 5. The bold, underlined regions of the amino acid sequence is the sequence of the C-terminal portion of the protein encoded by the unspliced form (SEQ ID NO: 7) that differs from that encoded by the spliced form, which is bold and underlined in Figure 5.

DETAILED DESCRIPTION

Since the ER stress signaling network plays a role in the pathogenesis of human diseases, it is important to monitor the ER stress level in mammalian cells. The present invention provides methods and reagents to quantify ER stress levels.

IRE1 is one of the most upstream components of ER stress signaling network and it is a sensor for ER stress. The present invention features quantifying IRE1 activation levels as a measure of ER stress. Because it is difficult to measure IRE1 activation levels directly, XBP-1 mRNA splicing levels, which precisely reflect IRE1 activation, are used to quantify the IRE1 activation levels. Spliced XBP-1 mRNA encodes the active XBP-1 transcription factor and activates the UPR. The invention features methods to quantify the activation level of XBP-1 using Reverse Transcriptase-PCR (RT-PCR). Primers are designed to amplify the region encompassing the splice junction of XBP-1 mRNA. The spliced form (the active form) of XBP-1 mRNA (cDNA) is smaller than the unspliced form (inactive form) by 26 base pairs. The size difference between the two forms can be visualized, for example, by electrophoresing the PCR products on an agarose gel.

Various aspects of the invention are described in further detail in the following subsections.

I. ER Stress and ER Stress Signaling Pathway Assays

The unfolded protein response (UPR) is a cellular adaptive response that counteracts ER stress. The UPR includes three different pathways to address ER stress: (1) gene expression, (2) translational attenuation, and (3) protein degradation. Inositol Requiring 1 (IRE1), an ER-resident transmembrane protein kinase, is one of the furthest upstream components of the UPR, and acts as a central regulator for UPR-specific downstream gene expression and apoptosis. At least in part, IRE1 acts by splicing a small intron from XBP-1 mRNA.

IRE1 and XBP-1 are crucial components of the UPR, and the expression levels of the active forms of XBP-1 and IRE1 can serve as markers for ER stress levels. It is difficult to directly measure the activation level of IRE1, because although activation of IRE1 by phosphorylation causes a shift to lower mobility on an SDS-polyacrylamide gel, the shift is very small and thus difficult to detect. To overcome this difficulty, the new methods use XBP-1 as a measure of ER stress level.

XBP-1 mRNA splicing levels can be detected using any method known in the art, e.g., Northern blotting, *in situ* hybridization (Parker & Barnes 1999 Methods in Molecular Biology 106:247–283), RNAse protection assays (Hod 1992, Biotechniques 13:852–854; Saccomanno et al. 1992 Biotechniques 13: 846–85), or reverse transcription polymerase chain reaction (RT-PCR) (Weis et al. 1992 Trends in Genetics 8:263–264).

In some embodiments, splice levels are detected using a nucleic acid probe, e.g., a labeled probe (a number of suitable labels are known in the art, including radioactive, fluorescent, spin, and calorimetric labels), that hybridizes to the intron that is removed from the XBP-1 sequence by splicing.

In some embodiments, XBP-1 splicing is detected using RT-PCR (reverse transcription-polymerase chain reaction, typically involving cDNA synthesis from a target mRNA by reverse transcription, followed by PCR amplification) and a pair of primers designed to amplify a region including the splice site. RT PCR methods are known in the art.

In some embodiments, the methods described herein measure splicing of XBP-1 by RT-PCR, optionally followed by Pst I digestion (See Examples 2-4). The mRNA and amino acid sequences for the spliced and unspliced forms of XBP-1 are shown in Figures 5 and 6, respectively. The underlined regions of each sequence correspond to (or are reverse complements of) primers for amplifying a region of the human XBP-1 mRNA that includes a splice junction. Additional primer pairs can readily be designed by the skilled artisan given the above sequences and primer design programs. The boxed region of the nucleotide sequence in Figure 6 is the sequences spliced out by IRE1. The splice junction is between nucleotides 506 and 507 in Figure 5. The bold, underlined regions of the amino acid sequence in Figure 5 is the sequence of the protein encoded by the spliced form that differs from that encoded by the unspliced form, which is bold and underlined in Figure 6.

In some embodiments, real-time PCR, e.g., as described in Bustin et al., J. Mol. Endocrinol. (2000) 25, 169–193, is used, for example, when more accurate quantification of splicing levels is required, e.g., where splicing levels are neither very high (e.g., most of the XBP-1 is in spliced form) nor very low (e.g., only some of the XBP-1 is in spliced form), but are in between (e.g., there is a more nearly balanced mixture of spliced and non-spliced XBP-1).

As noted above, any pairs of primers that can amplify the region of the target XBP-1 mRNA that includes a splice junction can be used. Exemplary sequences for primers are provided herein. Typically, the primer set will include a first primer that is identical to or complementary to a sequence that is 5' of the spliced intron region, and a second primer that is identical to or complementary to a sequence that is 3' of the spliced intron region, such that when the two primers are used in a polymerase chain reaction, a region of suitable size is obtained.

One of skill in the art will be able to design a suitable set of primers using the sequences of XBP-1 known in the art and provided herein.

In some embodiments, levels of ER stress are detected using a binding agent specific for the spliced or unspliced form of XBP-1 protein. In some embodiments, the binding agent is an antibody that is specific for the spliced or unspliced form, e.g., recognizes an epitope that is 3' of the splice site. For example, an antibody that is specific for the spliced form can recognize an epitope in SEQ ID NO:6; an antibody specific for the unspliced form can recognize an epitope in SEQ ID NO: 7. Such antibodies can include any form-specific antibody (e.g., a monospecific, or a recombinant or modified antibody), and includes antigen-binding fragments thereof (e.g., Fab, $F(ab')_2$, Fv or single chain Fv fragments). The antibodies can be of the various isotypes, including: IgG (e.g., IgG_1, IgG_2, IgG_3, IgG_4), IgM, IgA_1, IgA_2, IgD, or IgE. The antibody molecules can be full-length (e.g., an IgG_1 or IgG_4 antibody) or can include only an antigen-binding fragment (e.g., a Fab, $F(ab')_2$, Fv or a single chain Fv fragment). These include monoclonal antibodies, recombinant antibodies, chimeric antibodies, humanized antibodies, deimmunized antibodies, as well as antigen-binding fragments of the foregoing.

Antibodies (e.g., monoclonal antibodies from differing organisms, e.g., rodent, sheep, human) can be produced using art-recognized methods. Once the antibodies are obtained, the variable regions can be sequenced. The location of the CDRs and framework residues can be determined (see, Kabat, E.A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917). The light and heavy chain variable regions can, optionally, be ligated to corresponding constant regions. A light and the heavy immunoglobulin chains can be generated and co-expressed into the appropriate host cells.

Monoclonal anti-XBP-1 antibodies can be used in the methods described herein. Suitable monoclonal antibodies can be generated using techniques known in the art. Monoclonal antibodies can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and Milstein, Nature 256: 495 (1975). See generally, Harlow, E. and Lane, D. (1999) Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B

lymphocytes. A typical animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

Useful immunogens for the purpose of this invention include peptides comprising portions of XBP-1 that are unique to either the spliced or unspliced form of XBP-1, e.g., all or part of the sequences shown in SEQ ID NOs:6 (spliced form) and 7 (unspliced form). Human monoclonal antibodies (mAbs) directed against human proteins can be generated using transgenic mice carrying the human immunoglobulin genes rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L.L. et al. 1994 Nature Genet. 7:13-21; Morrison, S.L. et al. 1994 Proc. Natl. Acad. Sci. USA 81:6851-6855; Bruggeman et al. 1993 Year Immunol 7:33-40; Tuaillon et al. 1993 PNAS 90:3720-3724; Bruggeman et al. 1991 Eur J Immunol 21:1323-1326). Thus, the invention includes antibodies specific for a spliced or unspliced form of XBP-1.

The antibodies can be labeled to facilitate detection and quantification of XBP-1 splicing levels. Numerous suitable labels, and methods for labeling the antibodies, are known in the art. Examples of suitable labels include a fluorescent label, a biologically active enzyme label, a radioisotope (e.g., a radioactive ion), a nuclear magnetic resonance active label, a luminescent label, or a chromophore. In some embodiments, a labeled secondary antibody is used. See, e.g., Harlow and Lane, *Id.*

Quantitation can be performed using any method known in the art, including but not limited to fluorometry, gamma counting, scintillation counting, spectrophotometry, kinetic phosphorescence, or phosphorimaging. Computer-based methods can be used to facilitate analysis.

II. Uses

Quantifying or detecting ER stress is useful in any situation where it is suspected or has been determined that such stress may regulate a normal cellular phenotype (*e.g.*, regulate apoptosis) or cause or contribute to a disease phenotype (*e.g.*, a protein conformational disease phenotype). In mammalian cells, ER stress is regulated, at least in part, by an ER stress signaling pathway. This pathway is an evolutionarily conserved signaling network that is emerging as the major quality controller of newly synthesized proteins.

ER stress signaling is likely to be crucial for protein secretion and the development of secretory cells, such as plasma cells, adipocytes, and trophoblast cells in placenta. These data also suggest that defects in this signaling network can cause or contribute to human diseases, such as the diseases listed in Table 1, as well as others, including some forms of juvenile diabetes, inflammatory bowel disease, and cancers originated from secretory cells (e.g., breast cancer and prostate cancer).

The methods and reagents of the invention are suitable for use in methods to further study the role of ER stress in cellular processes such as apoptosis and contribution of such processes in a variety of ER stress diseases, and in methods of screening for compounds, e.g., drugs, useful in the treatment of such diseases. Thus, in some embodiments, the methods include providing a ER stress model system, e.g., a cell or animal model of an ER stress disease; optionally increasing levels of ER stress in the cells or animal (e.g., in at least some of the cells of the animal); contacting the cells with a test compound; and evaluating the levels of XBP-1 splicing in the cells in the presence and absence of the test compound, thus evaluating the effect of the compound on ER stress. Those compounds that produce a desired effect on ER stress, e.g., that substantially reduce ER stress (i.e., as measured by XBP-1 splicing levels), e.g., by at least about 20%, e.g., about 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, can be considered as candidate compounds and further evaluated for therapeutic activity using methods known in the art, e.g., administering the candidate compounds to an animal, e.g., an animal model of an ER stress disease, and evaluating an effect of the compound on the animal, e.g., therapeutic efficacy or toxicity.

In some embodiments, the system is an animal model of an ER stress disease, e.g., an animal model of diabetes (e.g., type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Cystic Fibrosis, familial hypercholesterolaemia and alpha1 antitrypsin deficiency, or

cells derived therefrom. Typically an ER stress disease can be induced in an otherwise healthy animal or cells by administering a compound known to cause ER dysfunction, e.g., by administering a sublethal dose of thapsigargin, tunicamycin (e.g., 0.25 – 1 mg/kg tunicamycin; see Zinszner et al., Genes and Dev. 1998, 12:982-995), or a proteosome inhibitor, e.g., lactacystin.

The new methods can be used in high-throughput screening methods, e.g., to screen a library of test compounds. For example, antibody-based or PCR-based high-throughput screening methods are known in the art and can be used to detect an effect on ER stress levels, e.g., by measuring XBA-I splicing levels. For high throughput screens, multi-well plates, e.g., plates with 96, 384, or more separate areas, e.g., wells, e.g., separated by a barrier, can be screened. Suitable plates are known in the art, and can be manufactured, modified, or are commercially available. In some embodiments, each area, e.g., each well, contains a unique compound, e.g., small molecule of known or unknown structure, or a pool of molecules of known or unknown structure.

The test compound library can be a library of compounds of related or unrelated structures. Such libraries are known in the art and are commercially available or can be synthesized using methods known in the art.

Libraries of test compounds, such as small molecules, are available, e.g., commercially available, or can be synthesized using methods known in the art. As used herein, "small molecules" refers to small organic or inorganic molecules. In some embodiments, small molecules useful for the invention have a molecular weight of less than 10,000 Daltons (Da). The compounds can include organic or inorganic naturally occurring or synthetic molecules including but not limited to soluble biomolecules such as oligonucleotides, polypeptides, polysaccharides, antibodies, fatty acids, etc.

The compounds can be natural products or members of a combinatorial chemistry library. A set of diverse molecules should be used to cover a variety of functions such as charge, aromaticity, hydrogen bonding, flexibility, size, length of side chain, hydrophobicity, and rigidity. Combinatorial techniques suitable for synthesizing small molecule compounds are known in the art, e.g., as exemplified by Obrecht, D. and Villalgrodo, J.M., Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon-Elsevier Science Limited (1998), and include those such as the "split and pool" or "parallel" synthesis

techniques, solid-phase and solution-phase techniques, and encoding techniques (see, for example, Czarnik, A.W., Curr. Opin. Chem. Bio., (1997) 1, 60). In addition, a number of compound, e.g., small molecule, libraries are commercially available.

Libraries and test compounds screened using the methods of the present invention can comprise a variety of types of compounds. A given library, for example, can comprise a set of structurally related or unrelated test compounds. In some embodiments, the compounds and libraries thereof can be obtained by systematically altering the structure of a first compound, e.g., a small molecule, e.g., using methods known in the art or the methods descried herein, and correlating that structure to a resulting biological activity, e.g., a structure-activity relationship study. As one of skill in the art will appreciate, there are a variety of standard methods for creating such a structure-activity relationship. Thus, in some instances, the work may be largely empirical, and in others, the three-dimensional structure of an endogenous polypeptide or portion thereof can be used as a starting point for the rational design of a test compound or compounds, e.g., a small molecule. For example, in one embodiment, a general library of small molecules is screened using the methods described herein.

Compounds identified as "hits" (e.g., compounds that decrease ER stress) in the first screen can be selected and systematically altered, e.g., using rational design, to optimize binding affinity, avidity, specificity, or other parameter. Such optimization can also be screened for using the methods described herein. Thus, in one embodiment, the invention includes screening a first library of compounds using the methods described herein, identifying one or more hits in that library, subjecting those hits to systematic structural alteration to create additional libraries of compounds structurally related to the hit, and screening the second library using the methods described herein.

In some embodiments, each well contains one or more unique text compounds, e.g., small molecules that are different from the test compounds in at least one of the other wells. In some embodiments, the multi-well plate also includes one or more positive and/or negative control wells. Negative control wells can contain, for example, no test compound other negative control. Positive control wells can contain, for example, compounds known to inhibit ER stress. In some embodiments, a number of multi-well plates, each comprising a unique set of small molecules, are screened. In this way, a library of test compounds in the hundreds, thousands, or millions can be screened for identification of ER stress reducing molecules.

The methods of the invention are also suitable for use in methods of diagnosing ER stress diseases, e.g., as described herein. For example, the methods and reagents can be used for diagnosing diabetes, e.g., Type 2 diabetes or certain forms of Type 1 diabetes, e.g., Wolcott-Rallison syndrome and Wolfram syndrome, as these diseases are believed to be caused, at least in part, by increased ER stress. More than one million people suffer from type 1 diabetes in the U.S. In this disease, insulin production is abnormally low because beta-cells in pancreatic islets are destroyed. Recent observations in the Akita diabetes model mouse (a C57BL/6 mouse with a mutation in insulin 2 gene; Oyadomari et al., J. Clin. Inv. 109:525-32 (2002); Urano et al., Science 287:664-6 (2000)) support the hypothesis that sufficient endoplasmic reticulum (ER) stress can cause beta-cell death.

It is believed that defects in the ER stress signaling network also cause or contribute to human diseases including many of the diseases listed in Table 1, as well as others, including inflammatory bowel disease, and cancers originated from secretory cells (*e.g.*, breast cancer and prostate cancer), as well as Parkinson's disease, cystic fibrosis, familial hypercholesterolaemia, alpha1-antitrypsin deficiency, and Alzheimer's disease. Thus, it is contemplated that the ER stress measurement methodologies described herein will also be useful in methods for diagnosing any of these diseases in patients. In some embodiments, the methods and reagents described herein can be used to diagnose the stage of a disease in patients. In some embodiments, the disease is multiple myeloma. Multiple myeloma is a cancer of plasma cells. ER stress signaling is important for the development of plasma cells. Thus, it is expected that ER stress levels will be very high in multiple myeloma cells, and higher stress levels is likely to correlate to more aggressive disease.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example 1: XBP-1 Splicing Assay

RNA from cells was reverse transcribed using Oligo-dT primer. PCR is performed using following primers:

Species	Sense (S) or Antisense (AS)	Sequence	SEQ ID NO:

[redacted]

Human	hXBP-1.1S	AAACAGAGTAGCAGCTCAGACTGC	8
Human	hXBP-1.2AS	TGGGCAGTGGCTGGATGAAAGC	9
Mouse	mXBP-1.3S	AAACAGAGTAGCAGCGCAGACTGC	10
Mouse	mXBP-1.6AS	CAGACAATGGCTGGATGAAAGC	11
Rat	rXBP-1.3S	AAACAGAGTAGCAGCACAGACTGC	12
Rat	mXBP-1.6AS	CAGACAATGGCTGGATGAAAGC	13

These primers amplify a 768-base pair PCR product for human, a 774-base pair PCR product for mouse, and a 774-base pair PCR product for rat from the unspliced XBP-1, and 742-base pair (human) and 748-base pair (mouse, rat) PCR products from the spliced form. These primers were designed to amplify the region encompassing the splice junction of XBP-1 mRNA.

Reverse Transcriptase-PCR (RT-PCR) was performed using mRNA isolated using standard methods from a wild-type mouse fibroblast cell line and Ire1α:Ire1β double knock-out cell line. The cells were treated with tunicamycin or thapsigargin for 4 or 8 hours. Tunicamycin causes ER stress experimentally by blocking N-linked glycosylation, which is a crucial step for protein folding in the ER. Thapsigargin also induces ER stress experimentally by altering Calcium ion concentrations in the ER.

The results are illustrated in Figure 1B. The 26 base pair size difference between the two forms, spliced and unspliced, was visualized by running the PCR product on 2.5% agarose gel (Figure 1B). The thermal cycle reaction was performed as follows: 94°C for 3 minutes, followed by 35 cycles at 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes. RT-PCR analysis detected predominantly smaller fragment corresponding to active form (spliced form) of XBP-1 mRNA in wild-type cell line treated with tunicamycin or thapsigargin. In contrast, the same analysis detected only inactive form of XBP-1 mRNA in Ire1α-/-:Ire1β-/- double knock-out cell line (Figure 1B).

Example 2: XBP-1 Splicing Assay with Pst I Digestion

A Pst I restriction site is removed by IRE1-mediated cleavage and splicing of the mRNA, thus, the results of the experiment described in Example 1 can also be achieved using an intermediate step of Pst I cleavage to facilitate distinguishing between spliced and unspliced XBP-1. Pst I digestion of the spliced form of XBP-1 yields a 768-base pair fragment for human, 774-base pair fragment for mouse and rat. The unspliced forms of XBP-1 yield 285 base pair

and 483 base pair fragments for human, 291 base pair and 483 base pair fragments for mouse and rat.

RT-PCR performed as described in Example 1 was followed by Pst I digestion, and the digested products were visualized on a 2% agarose gel. Since the intron removed by IRE1-mediated splicing contains the Pst I site, the spliced form (the active form) of XBP-1 mRNA (cDNA) loses its Pst I site after IRE1 processing. Pst I digestion of RT-PCR product produces undigested larger fragment corresponding to the active form (spliced form, no Pst I site) of XBP-1 mRNA and two smaller, digested fragments corresponding to the inactive form (unspliced form, which retains the Pst I site) (Figure 2A). Pst I digestion of RT-PCR product generated as described above detected predominant non-digested fragment corresponding to active form (spliced form) of XBP-1 mRNA in wild-type cell line treated with tunicamycin or thapsigargin. In contrast, the same analysis detected only inactive form of XBP-1 mRNA in Ire1α-/-:Ire1β-/- double knock-out cell line (Figure 2B).

Example 3: ER stress Signaling is Activated in Islet Cells under Physiological Conditions

To determine whether ER stress signaling is activated in islet cells under physiological conditions, XBP-1 splicing was monitored in freshly isolated mouse islet cells, using the methods described above in Example 2. The results are shown in Figure 3. High levels of XBP-1 mRNA splicing were detected in the islet cells. Dithiothreitol (DTT) treatment enhanced the XBP-1 splicing. It is known that DTT blocks disulfide bond formation experimentally, resulting in ER stress. These results illustrate that XBP-1 splicing, and hence ER stress, occurs in islet cells under physiological conditions. This demonstrates that the methods described herein can be successfully used to detect and measure ER stress under physiological conditions; in addition, as the islet cells secrete insulin, this demonstrates that ER stress may play a role in the etiology of diabetes.

Example 4: Insulin-2 Mutation in the Akita Mouse Causes ER Stress in MIN6 Cells

The Akita diabetes model mouse is a C57BL/6 mouse which is heterozygous for a mutation in insulin 2 gene. This mutation results in an amino acid substitution, cysteine 96 to tyrosine. Cysteine 96 is involved in the formation of one of the two disulfide bonds between the A and B chains of mature insulin. It is likely that this mutation causes incorrect folding of

insulin precursor in the endoplasmic reticulum (ER) of pancreatic beta-cells. Diabetes in the Akita mouse is accompanied by neither obesity nor insulitis. These mice spontaneously develop diabetes with dramatic reduction in beta-cell mass. Symptoms include hyperglycemia, hypoinsulinemia, polydipsia, and polyuria, beginning around 4 weeks of age. This condition in the Akita mouse is termed diabetes.

Since the phenotype of the Akita mouse is caused by a mutation which can cause conformational changes in the insulin 2 (Ins2) gene product (Wang et al., J., 1999. J. Clin. Invest. 103:27-37), it is hypothesized that pancreatic cells in Akita mice are under ER stress, and this stress can cause beta cell death. To test this hypothesis, XBP-1 splicing levels were measured in mouse insulinoma cells (MIN6 cells) expressing either an Ins2 gene with the Akita mutation or a wild-type insulin-2 gene. The MIN6 cells were cultured in 10 cm collagen-coated dishes in DMEM supplemented with 25 mM glucose and 15% FCS. Plasmids encoding the wild-type or mutant Ins-2 genes were transfected into the cells using Fugene™ transfection reagent following the manufacturer's instructions (Roche, Basel, Switzerland).

The results are shown in Figure 4. High XBP-1 splicing levels, which reflected high ER stress levels, were detected in the MIN6 cells expressing mutant insulin 2 gene. This indicates that the methods described herein can be used to detect differences in ER stress levels correlating with disease states.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of evaluating endoplasmic reticulum stress (ER stress) levels in a cell or biological sample, the method comprising detecting an IRE1 activation level in the cell or biological sample by detecting an XBP-1 splicing level, wherein the IRE1 activation level correlates with ER stress, thus evaluating the ER stress levels in the cell or biological sample.

2. The method of claim 1, further comprising quantifying an XBP-1 splicing level.

3. The method of claim 1, wherein the XBP-1 splicing level is determined by:

amplifying an XBP-1 mRNA region which includes a splice site, or portion thereof; and

detecting the size of the amplified mRNA, wherein the size is indicative of spliced or unspliced mRNA; such that the XBP-1 splicing level is determined.

4. The method of claim 3, wherein the amplified mRNA is subjected to restriction enzyme digestion to facilitate detection of spliced or unspliced mRNA.

5. The method of claim 4, wherein the restriction enzyme digestion is Pst I digestion.

6. The method of any one of the preceding claims, wherein the ER stress level is quantified in a cell.

7. The method of any one of the preceding claims, wherein the ER stress level is quantified in a mammalian cell.

8. The method of any one of the preceding claims, wherein the ER stress level is quantified in a human cell.

9. The method of claim 8, wherein the cell is a pancreatic beta cell.

10. The method of any one of claims 1-5, wherein the ER stress level is quantified in a cell extract.

11. A method of diagnosing an ER stress disease in a subject, the method comprising quantifying the level of ER stress in a cell or biological sample isolated from the subject using a method according to any one of the preceding claims, wherein an increased level of ER stress is indicative of an ER stress disease.

12. A method of monitoring the progression of an ER stress disease in a subject, the method comprising quantifying the level of ER stress in a cell or biological sample isolated from the subject at sequential time points using a method according to any one of the preceding claims, wherein a change in level of ER stress is indicative of the progress of the ER stress disease.

13. The method of claim 11 or 12, wherein the ER stress disease is diabetes.

14. A method of screening a test compound for an effect on ER stress, the method comprising:

providing an ER stress model system *(e.g., a system comprising a cell expressing IRE1 and XBP-1, e.g., a cultured cell or animal)*;

optionally, increasing levels of ER stress in the system;

contacting the system with a test compound; and

evaluating the levels of XBP-1 splicing in the system in the presence and absence of the test compound,

wherein an increase in XBP-1 splicing indicates an increase in ER stress, and a decrease in XBP-1 splicing indicates a decrease in ER stress, thereby evaluating the effect of the compound on ER stress.

15. The method of claim 14, wherein the ER stress model system is a cell or animal model of an ER stress disease.

16. The method of claim 14, wherein increasing levels of ER stress in the system comprises increasing levels of ER stress in the cell or in at least one cell in the animal.

17. The method of claim 14, wherein levels of ER stress in the system are increased by contacting the system with an agent that increases levels of ER stress.

18. The method of claim 17, wherein the agent that increases levels of ER stress is thapsigargin or tunicamycin.

19. The method of claim 14, wherein the effect is a decrease in levels of ER stress.

20. A kit for quantifying ER stress, comprising:

primers for amplifying a region of XBP-1 mRNA which includes a splice site, or portion thereof, and

instructions for use.

21. The kit of claim 20, further comprising a suitable control.

ABSTRACT

The present invention provides methods and reagents to quantify endoplasmic reticulum stress (ER stress) levels. Methods for quantifying ER stress in mammalian cells are exemplified.

Figure 1A.

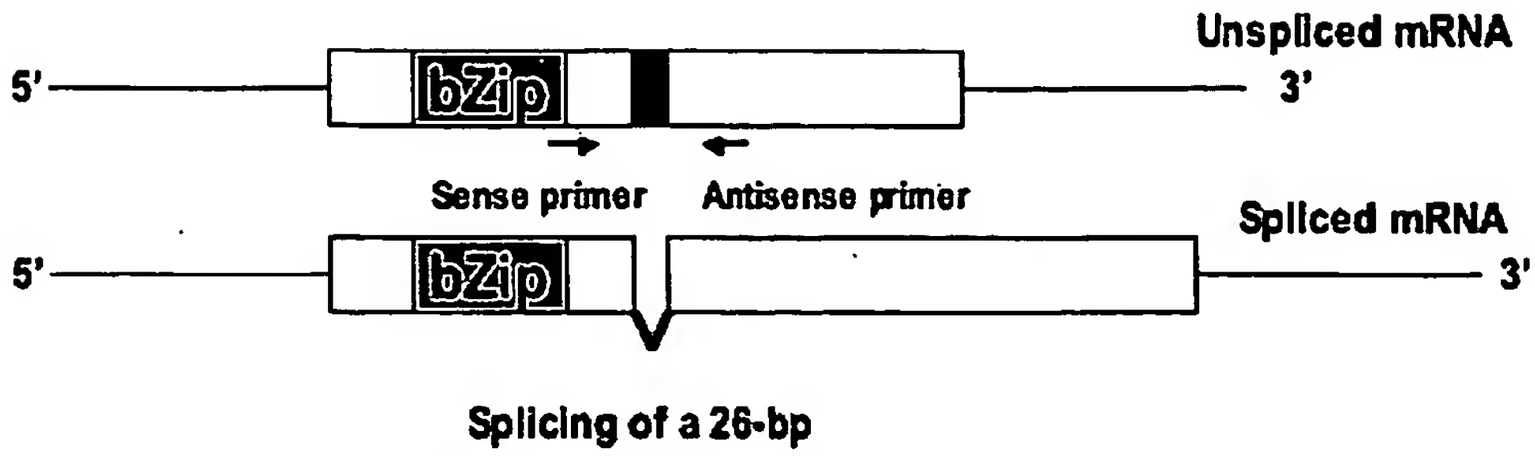


Figure 1B.

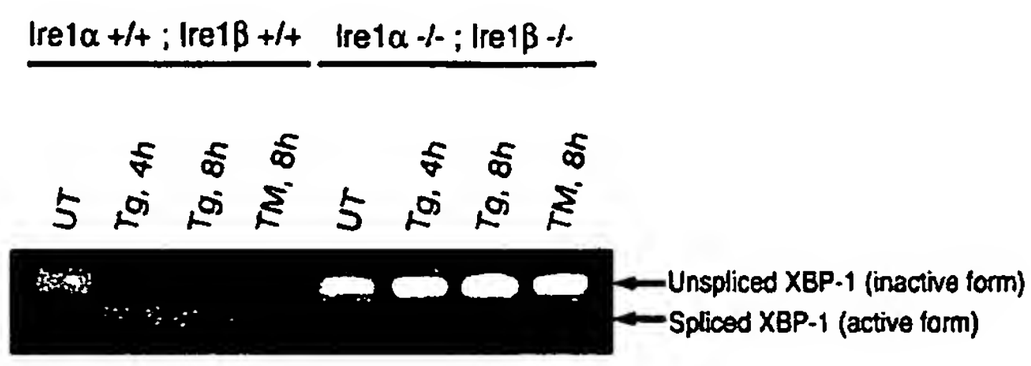
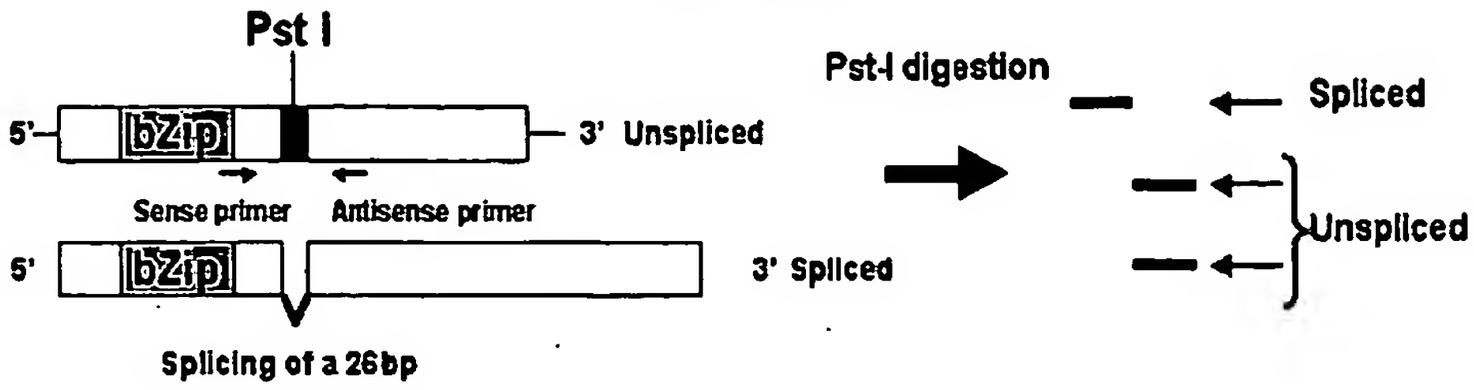


Figure 2A.



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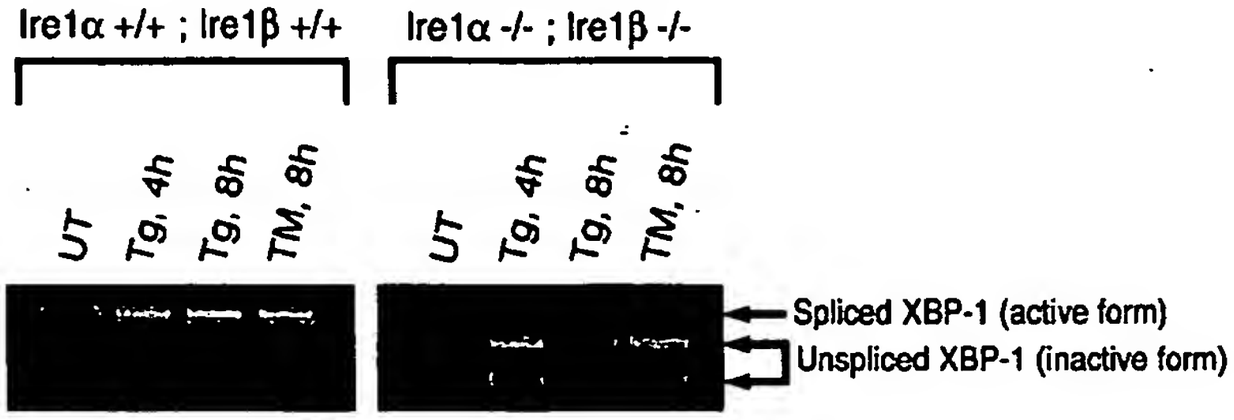

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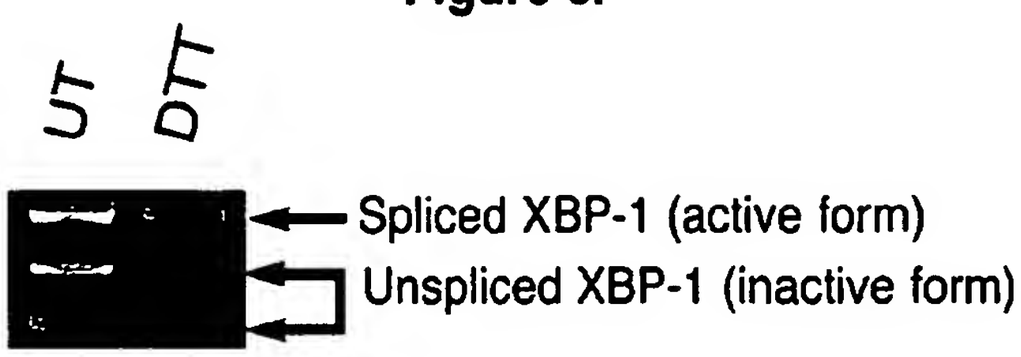

Figure 4.

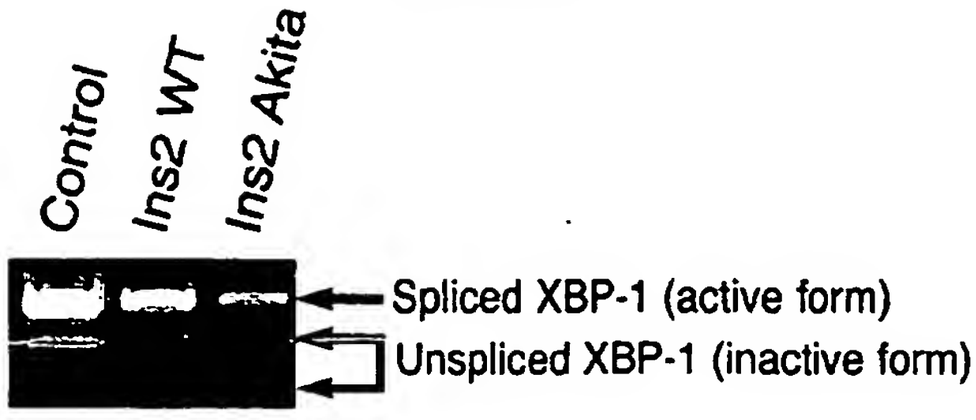

Figure 5

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  AUTHORS   Yoshida,H., Matsui,T., Yamamoto,A., Okada,T. and Mori,K.
  TITLE     XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER
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  JOURNAL   Submitted (18-DEC-2001) Hiderou Yoshida, Kyoto University, Graduate
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Figure 6

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VERSION     AB076383.1  GI:18148379
KEYWORDS    .
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REFERENCE   1
  AUTHORS   Yoshida,H., Matsui,T., Yamamoto,A., Okada,T. and Mori,K.
  TITLE     XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER
            Stress to Produce a Highly Active Transcription Factor
  JOURNAL   Cell (2001) In press
REFERENCE   2  (bases 1 to 1787)
  AUTHORS   Yoshida,H. and Mori,K.
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  JOURNAL   Submitted (18-DEC-2001) Hiderou Yoshida, Kyoto University, Graduate
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